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TITLE: Neuroinflammatory Pathobiology in Gulf War Illness: Characterization with an Animal Model

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14. ABSTRACT

Gulf War Illness (GWI) is a multi-symptom syndrome with features of an inflammatory response due to infection or injury, findings suggestive of a chronic neuroimmune/neuroinflammatory disorder. *Our overarching hypothesis is that exposure to GWI-relevant compounds lead to enhanced and/or prolonged expression of proinflammatory mediators in the brain.* Our overall objective is to establish a neuroinflammatory model of GWI-related exposures, to define the contribution of high physiological stress, and to assess the potential for pharmacotherapy to ameliorate these effects. The results are suggestive of a possible critical and as yet unrecognized interaction between the stressful environs of the GW theater and agent exposure(s) unique to this war, exposures in which the CNS is primed to amplify future exposures to pathogens, injury or toxicity. Recently it has been shown that stress-induced augmentation of neuroinflammation induced by a positive control compound persists for 30 days after stress presentation, and that repeated intermittent stress presentations have a cumulative effect on the neuroinflammation. Such occurrences could potentially result in prolonged episodes of sickness behavior that would simulate GWI well.

15. SUBJECT TERMS

Gulf War Illness, chronic neuroinflammation, diisopropyl phosphorofluoridate, physiological stress, minocycline, cytokines, hippocampus

Table of Contents

	<u>Page</u>
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	10
Reportable Outcomes.....	11
Conclusion.....	11
References.....	N/A
Appendices.....	12

Introduction

Gulf War Illness (GWI) is a multi-symptom disorder with features characteristic of “sickness” behavior including cognitive impairment, fatigue, depression, sleep disruption, and gastrointestinal and dermatological problems. Sickness behavior, a normal manifestation of an inflammatory response due to infection or injury, resolves when homeostasis is restored, but in GWI the symptoms persist, findings suggestive of a heightened or chronic neuroimmune/neuroinflammatory disorder. The expression of proinflammatory cytokines and chemokines are the basis of sickness behavior and are the key elements of inflammation. Notably, low level inhalation exposure of sarin in experimental animals, the nerve agent implicated in GWI, causes neuroinflammation; further, the sarin surrogate diisopropyl phosphorofluoridate (DFP) increases proinflammatory cytokine expression in multiple brain regions. Finally, the stress of the war theater may have affected the blood brain barrier, allowing GWI-relevant agents access to the CNS. A role for stress in GWI is bolstered by our finding of increased proinflammatory cytokine expression in brain when DFP is preceded by a week of treatment with the rodent-specific stress hormone, corticosterone (CORT). The above observations suggest a possible critical and unrecognized link between the stressful environment of the Gulf War (GW) theater, agent exposure(s) unique to this war, and a resulting adverse heightened neuroinflammatory outcome. *Our overarching hypothesis is that exposure to GWI-relevant compounds leads to enhanced and/or prolonged expression of proinflammatory mediators in the brain.* Our data also lead us to hypothesize that brief high physiological levels of the stress hormone, cortisol (CORT in the rodent), greatly exacerbate the neuroinflammatory effects of GWI-related exposures, and that the FDA-approved anti-inflammatory, minocycline, can ameliorate heightened neuroinflammation. *Thus, our overall objective is to establish a neuroinflammatory model of GWI-related exposures, to define the contribution of high physiological stress in the initiation, strength, and duration of the neuroinflammation observed, and to assess the potential for currently available pharmacotherapy to ameliorate these effects with the ultimate goal of treating veterans suffering from GWI.*

Body

The accomplishments associated with the third year of the project are outlined below with linkage to each task in the detailed Statement of Work.

Timeline: Because work at the Centers for Disease Control and Prevention could not be initiated until establishment of the DOD-initiated and UIC-approved CRADA, funds were not available and work on the project did not commence until Oct. 1, 2010, the beginning of the fiscal year (after late July 2010 approval of the CRADA between UIC and CDC). Thus, the progress reported to date has occurred during the first 35 months of the project.

Tasks 3 & 6. Determine if Chronic CORT Influences the Impact of LPS or DFP Challenge in PB- and DEET-treated mice. The 15 day post-CORT time point has been completed for the CORT & LPS and CORT & DFP combined treatments. Tissue from animals at the 90 and 180 day post-CORT points has been harvested and analyses of the samples are on schedule. Preliminary

results are shown in figures 1 through 3. In the 15 day post-CORT experiments we found that chronic CORT given in the drinking water for 7 days caused exacerbated and prolonged neuroinflammation after a single LPS administration 24 hours after the cessation of CORT treatment. We have found that this priming of the CNS neuroinflammatory response can last at least 30 days after LPS. In addition, after CORT treatment for 7 days mice were left untreated for 30 days and then given a single systemic dose of LPS. At 6 hours after LPS administration, the mice previously exposed to CORT in the drinking water for 7 days showed significantly higher expression of proinflammatory cytokines and chemokines in the hippocampus (figure 1) and cortex (figure 2). This CORT priming effect was no longer present when mice were left untreated for 180 days (figure 3).

Another group of mice were dosed with CORT in the drinking water for 1 week out of each month for 180 days. At 6 h after LPS administration on day 180 the repeated intermittent CORT regimen caused significant exacerbation of LPS-induced neuroinflammation (figure 3). The degree to which this CORT dosing regimen exacerbated the expression of proinflammatory cytokines and chemokines is in some cases more than 1000 fold over that seen at the 15 day time point.

Task 8. Determine the functional consequences of exposure to GWI-Relevant Agents (Meets Aims 1-3) Efforts to provide functional correlates to the neuroinflammation resulting from various combinations of chronic CORT and inflammagen cholinesterase inhibitors through the implementation of synaptic plasticity paradigms are ongoing at the University of Illinois College of Medicine using the mouse model and recording in anesthetized intact animals. Input-output (I/O) curves are evaluated in each animal along with paired-pulse functions and induction of hippocampal long term potentiation (LTP) – an electrophysiological correlate of learning/memory. These assessments are performed in CORT-treated mice 15 or 90 days after challenge with DFP or LPS to determine whether neuronal excitability parallels the neuroinflammatory changes that are occurring. LTP from mice treated in the same way but also given minocycline are being evaluated as well. Mice treated with CORT, PB & DEET and challenged with LPS or DFP are being evaluated with experimental parameters mirroring those employed in studies to fulfill Tasks 5 & 6. This work is ongoing, but there are no completed group results to present at this time.

Task 9. Disseminate information. Some of these findings were summarized in abstract form and presented at the Annual Meeting for the Society of Toxicology in San Antonio, TX in March, 2013.

More recent work has been summarized in abstract form and will be presented at the Annual Meeting for the Society for Neuroscience in San Diego, CA in November 2013.

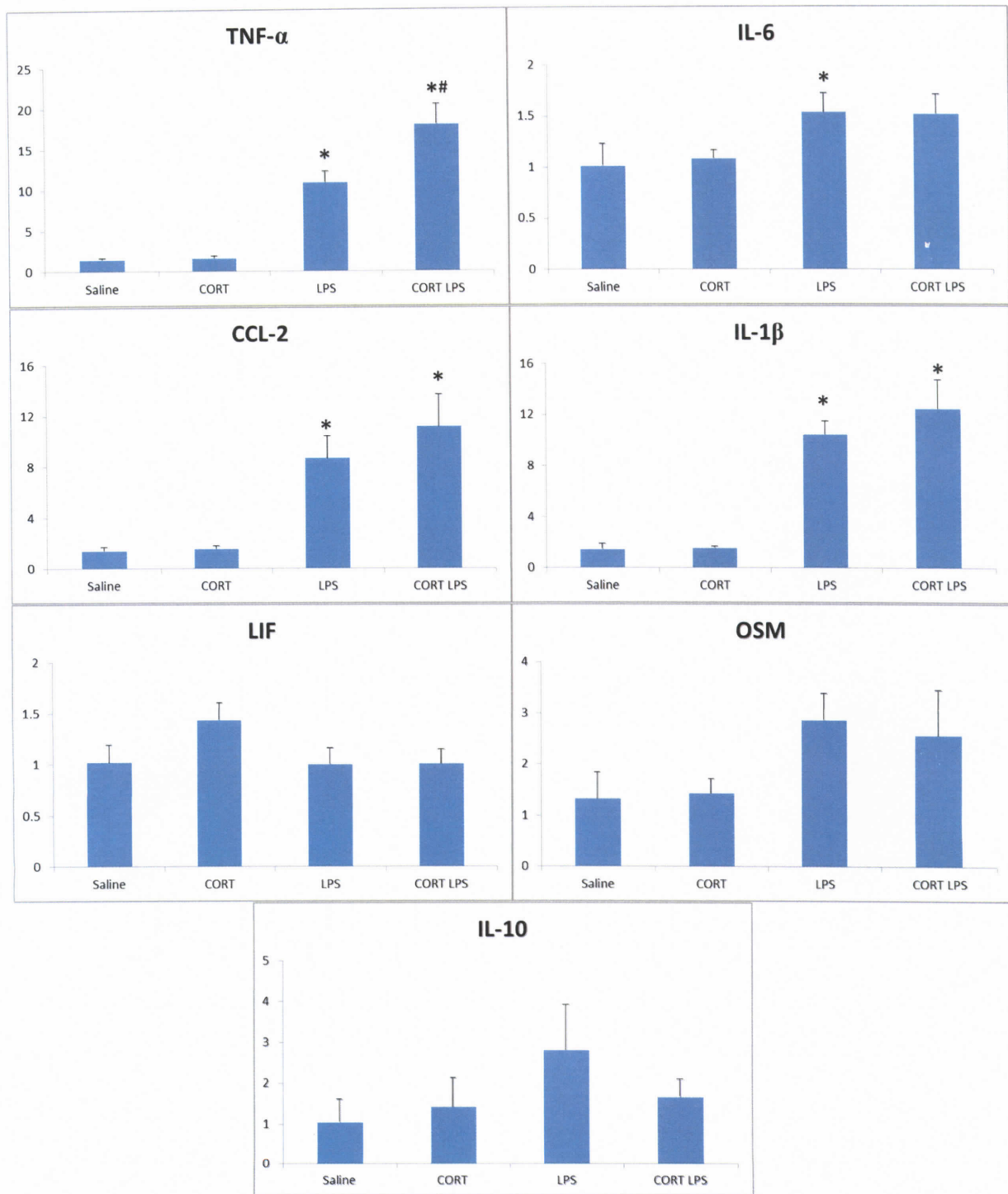


Figure 1: Lasting priming effect of chronic CORT when LPS is administered 30 days after cessation of CORT. Mice were treated with CORT (200 ug/ml drinking water) for 7 days followed by LPS (2 mg/kg, s.c.) as a single dose 30 days after cessation of CORT treatment. At 6 hours post LPS exposure, mice were sacrificed and total RNA was extracted from the hippocampus. Real time PCR analysis was performed for TNF- α , IL-6, CCL-2, IL-1 β , LIF, OSM and IL-10. Bars represent mean \pm SEM (N=5 mice/group.) Statistical significance of $p < 0.05$ by ANOVA with Fisher LSD post hoc analysis is denoted as * vs Saline and # vs LPS.

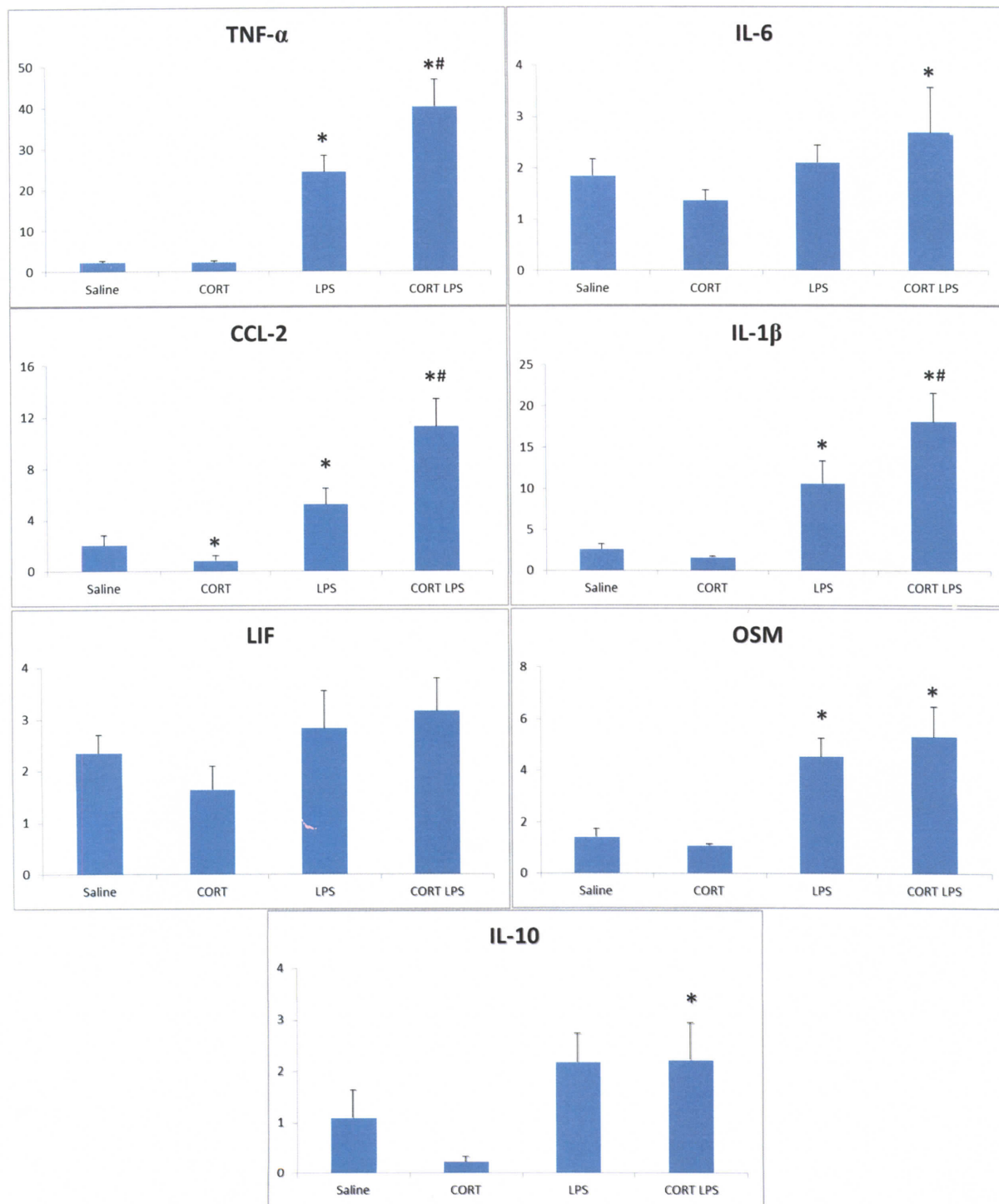


Figure 2: Lasting priming effect of chronic CORT when LPS is administered 30 days after cessation of CORT. Mice were treated with CORT (200 ug/ml drinking water) for 7 days followed by LPS (2 mg/kg, s.c.) as a single dose 30 days after cessation of CORT treatment. At 6 hours post LPS exposure, mice were sacrificed and total RNA was extracted from the cortex. Real time PCR analysis was performed for TNF- α , IL-6, CCL-2, IL-1 β , LIF, OSM and IL-10. Bars represent mean \pm SEM (N=5 mice/group.) Statistical significance of $p < 0.05$ by ANOVA with Fisher LSD post hoc analysis is denoted as * vs Saline and # vs LPS.

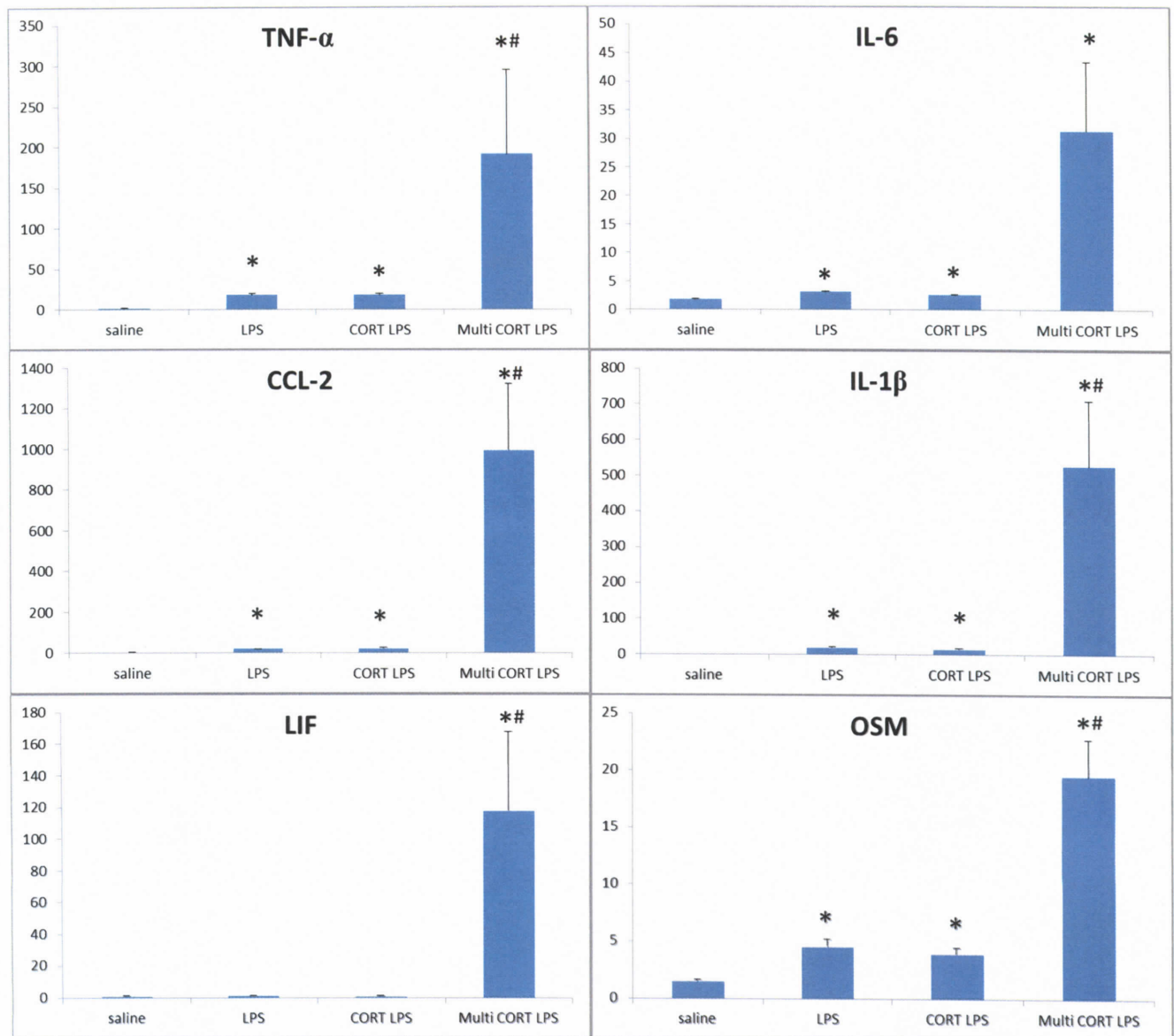


Figure 3: Cumulative effect of multiple CORT exposures over 6 months on LPS induced neuroinflammation. In the CORT LPS group mice were treated with CORT (200 ug/ml drinking water) for 7 days followed by LPS (2 mg/kg, s.c.) as a single dose 180 days after cessation of CORT treatment. The Multi-CORT LPS group comprised CORT treatments lasting 7 days once per month for 6 months with a single dose of LPS (2 mg/kg, s.c.) at 180d. At 6 hours post LPS exposure, on day 180, mice were sacrificed and total RNA was extracted from the cortex. Real time PCR analysis was performed for TNF- α , IL-6, CCL-2, IL-1 β , LIF, OSM and IL-10. Bars represent mean \pm SEM (N=5 mice/group.) Statistical significance of $p < 0.05$ by ANOVA with Fisher LSD post hoc analysis is denoted as * vs Saline and # vs LPS.

Key Research Accomplishments

1. Demonstration that prior administration of high physiological stress levels of CORT augment the neuroinflammatory effect of LPS out to 30 days after cessation of CORT treatment. That is, the CORT priming effect on LPS-induced neuroinflammation endures for at least a few weeks.
2. Demonstration that repeated intermittent exposures to high physiological stress levels of CORT have a cumulative effect on the CNS neuroinflammatory response. That is, multiple chronic exposures to CORT appear to have a potentiating effect on LPS-induced neuroinflammation.

Reportable Outcomes

2011 Society for Neuroscience Abstract: Chronic exposure to glucocorticoids, PB and DEET primes the neuroinflammatory response to the nerve agent DFP in a potential model for Gulf War Illness. K.A. Kelly¹, D.B. Miller¹, S.M. Lasley², and J.P. O'Callaghan¹. 1. CDC-NIOSH, Morgantown, WV; 2. University of Illinois College of Medicine, Peoria, IL.

This abstract was selected by the Society for Neuroscience as being newsworthy. A lay version of the abstract's content and significance of the research was available to the press during the Annual Meeting in November, 2011.

2013 Society of Toxicology Abstract: Glucocorticoid exposure primes the neuroinflammatory response to the nerve agent DFP in a model of Gulf War Illness. K.A. Kelly¹, D.B. Miller¹, S.M. Lasley², and J.P. O'Callaghan¹. 1. CDC-NIOSH, Morgantown, WV; 2. University of Illinois College of Medicine, Peoria, IL.

2013 Society for Neuroscience Abstract: The anti-inflammatory glucocorticoid, corticosterone, markedly augments systemic inflammatory responses to the nerve agent DFP in a mouse model of Gulf War Illness. K.A. Kelly¹, D.B. Miller¹, S.M. Lasley², M.A. Fletcher³, Z. Barnes³, and J.P. O'Callaghan¹. 1. NIOSH, Centers For Disease Control and Prevention, Morgantown, WV; 2. Univ. of Illinois College of Medicine, Peoria, IL; 3. NOVA Southeastern Univ., Miami, FL.

Conclusions to date:

Our findings are suggestive of a possible critical and as yet unrecognized interaction between the stressful environs of the GW theater and agent exposure(s) unique to this war, exposures in which the CNS is primed by stress to amplify future exposures to pathogens, injury or toxicity. Such occurrences could potentially result in prolonged episodes of sickness behavior similar to those of GWI.

Appendices

- 1: Task Summary Tables
- 2: Society for Neuroscience abstract 2011
- 3: Society of Toxicology abstract 2013
- 4: Society for Neuroscience abstract 2013

#1

CDMRP data completed summary as of 8/13

Gulf War assay completion summary as of 8/13

Date	Challenge	Time	pt	Mino	PCR-brain area	PCR	GFAP
2/24/2011	15 day	2 hr			Hippocampus, Cortex CB, Hypo, OB, Str &	OSM,GFAP,IL6,CCL2, TNF, LI1B, Lif, IL10	
2/22/2011	15 day	6 hr			Liver	OSM,GFAP,IL6,CCL2, TNF, LI1B, Lif, IL10	
4/5/2011	15 day	12 hr			Hip, Str, Cortex, Liver	OSM,GFAP,IL6,CCL2, TNF, LI1B, Lif, IL10	
4/7/2011	15 day	72 hr					Hippo, Cortex, Str
8/22/2011	15 day	6 hr		X	Hip, Ctx		
8/25/2011	15 day	12 hr		X	Ctx		
	180 day	6 hr			Ctx		

CORT=corticosterone;(200mg/L) for days 7-14
 PB=pyridostigmine bromide;PB(2.5 mg/kg/day, s.c.)days 1-14
 D=diethyl-m-toluidide;DEET(30 mg/kg/day, s.c.) for days 1-14
 LPS=lipopolysaccharide; (2 mg/kg, s.c.) day 15
 DFP=diisopropyl phosphorofluoridate;(4mg/kg/day,i.p.)day 15
 Mino=minocycline; (100mg/kg, s.c.) days 1-14

Ctx	Hip	Str	Liver	OB, Hypo, CB
2h	2h			
6h	6h	6h	6h	6h
12h	12h	12h	12h	
mino 6h	mino 6h			
mino 12h				
180d 6h				

Hip;Hippocampus, Ctx;Cortex, Str;Striatum, Cb;Cerebellum, OB;Olfactory Bulb, Hypoth;Hypothalamus

#2 Society for Neuroscience Abstract 2011

Chronic exposure to glucocorticoids, PB and DEET primes the neuroinflammatory response to the nerve agent DFP in a potential model for Gulf War Illness.

K.A. Kelly¹; D.B. Miller¹; S.M. Lasley²; J.P. O'Callaghan¹, 1. CDC-NIOSH, Morgantown, WV; 2. University of Illinois College of Medicine, Peoria, IL.

We have shown that chronic exposure to the glucocorticoid, corticosterone (CORT), at levels associated with high physiological stress, can prime the CNS proinflammatory response to neurotoxic exposures and systemic inflammation. Such neuroinflammatory events can be associated with sickness behavior. Gulf War (GW) Illness is a multi-symptom disorder with features characteristic of persistent sickness behavior. GW veterans were exposed to the stresses of war, prophylactic treatment with the reversible acetylcholinesterase (AChE) inhibitor pyridostigmine bromide (PB), the insect repellent DEET and, potentially, the nerve agent, sarin. These combined exposures were designed to mimic the conditions existing in theater during the 1991 GW. Here we examined whether CORT exposures primed the CNS to mount neuroinflammatory responses to GW exposures. Male C57BL/6 mice were treated with chronic (14 days) PB (2 mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.); as well as subchronic exposure to CORT (200 µg/ml in the drinking water on days 7-14) followed by acute exposure (day 15) to the sarin surrogate and irreversible AChE inhibitor, diisopropyl phosphorofluoridate; (DFP) (4 mg/kg, i.p.) or known inflammogen, lipopolysaccharide (LPS) (2 mg/kg, s.c.). We found that DFP alone, as well as LPS alone, caused a marked neuroinflammation as assessed by qRT-PCR of IL1 β , TNF α , IL6, CCL2, LIF and OSM. Chronic pretreatment with high physiological levels of CORT and/or PB and DEET greatly augmented (up to 500-fold) the neuroinflammatory response to LPS and DFP in hippocampus, cortex and striatum. Our findings are suggestive of a possible critical and as yet unrecognized interaction between the stressful environs of the GW theater and agent exposure(s) unique to this war, exposures in which the CNS is primed to amplify future exposures to pathogens, injury or toxicity. Such occurrences could potentially result in prolonged episodes of sickness behavior. (Supported by CDMRP)

#3 Society of Toxicology abstract 2013

Glucocorticoid exposure primes the neuroinflammatory response to the nerve agent DFP in a model of Gulf War Illness

KA Kelly¹, DB Miller¹, SM, Lasley² and JP O'Callaghan¹

1 CDC-NIOSH, Morgantown, WV

2 University of Illinois College of Medicine, Peoria, IL

We have shown previously that chronic exposure to the glucocorticoid corticosterone, (CORT), at levels associated with high physiological stress, can prime the CNS proinflammatory response to neurotoxic insults. Persistent sickness behavior, a prominent component of Gulf War (GW) Illness, is associated with neuroinflammation. Veterans of the 1991 GW were exposed to the stresses of war, prophylactic treatment with the reversible acetylcholinesterase (AChE) inhibitor, pyridostigmine bromide (PB), the insect repellent, DEET, and, potentially, acutely to the nerve agent sarin. Previously, we showed that subchronic CORT pretreatment primed the CNS to mount a neuroinflammatory response to these GW exposures when provoked 24 h after the termination of CORT treatment. Here, we investigated the persistence of this priming effect on neuroinflammation and the minimal amount of CORT required to produce the priming response. Male C57BL/6 mice were pretreated with chronic (14 days) PB (2mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.), as well as subchronic CORT (200 µg/ml in drinking water on days 7-14) or acute CORT (20 mg/kg, s.c., 2 injections, 7 h interval on day 14) exposures. Acute doses (days 15 or 45) of the sarin surrogate and irreversible AChE inhibitor diisopropyl phosphorofluoridate (DFP, 4 mg/kg, i.p.) or known inflammogen lipopolysaccharide (LPS, 2 mg/kg, s.c.) were used to provoke neuroinflammation. We found that both acute and chronic CORT exposure greatly augmented neuroinflammation in response to LPS and DFP. Marked neuroinflammation in response to DFP was found even when the exposure was 30 days after the cessation of the subchronic CORT treatment (a time point equivalent to 6 years in humans). Our findings are suggestive of a possible critical and yet unrecognized link between the stressful environs of the 1991 GW theater and agent exposure(s) unique to this war, exposures in which the CNS is primed to amplify future exposure to pathogens, injury or toxicity. (Supported by CDMRP W81XWH-09-2-0098)

#4 Society for Neuroscience abstract 2013

The anti-inflammatory glucocorticoid, corticosterone, markedly augments systemic inflammatory responses to the nerve agent DFP in a mouse model of Gulf War Illness

K. A. KELLY¹, D. B. MILLER¹, S. M. LASLEY², M. A. FLETCHER³, Z. BARNES³, *J. P. O'CALLAGHAN¹;

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We have shown that chronic exposure to the glucocorticoid corticosterone (CORT), at levels associated with high physiological stress, can exacerbate CNS proinflammatory responses to neurotoxic insults. Persistent sickness behavior, a prominent component of Gulf War (GW) Illness, is associated with neuroinflammation. Veterans of the 1991 GW were exposed to the stresses of war, prophylactic treatment with the reversible acetylcholinesterase (AChE) inhibitor pyridostigmine bromide (PB), the insect repellent DEET, and potentially acutely to the nerve agent sarin. Previously, we found the sarin surrogate diisopropyl phosphorofluoridate (DFP) to induce a neuroinflammatory response in multiple brain areas of the mouse. Attempts to suppress this response with subchronic pretreatment with the anti-inflammatory CORT unexpectedly resulted in an exacerbated "priming" of the CNS neuroinflammatory response. Here, we investigated the inflammatory effects systemically. Male C57BL/6 mice were pretreated with chronic (14 days) PB (2mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.), as well as subchronic CORT (200 µg/ml in drinking water on days 7-14) exposure. Acute doses (at day 15) of the sarin surrogate and irreversible AChE inhibitor diisopropyl phosphorofluoridate (DFP, 4 mg/kg, i.p.) or known inflammogen lipopolysaccharide (LPS, 2 mg/kg, s.c.) were used to provoke neuroinflammation. Brain regions and liver samples were assessed by RT-PCR for interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), IL-6, chemokine (C-C motif) ligand 2 (CCL-2), leukemia inhibitory factor (LIF) and Oncostatin M (OSM). Serum samples were tested by chemiluminescent ELISA for concentrations of cytokines: IL-1α, -1β, -2, -4, -5, -6, -10, -12, TNF-α, keratinocyte chemoattractant (KC), macrophage inflammatory protein-2 (MIP-2), and interferon-gamma (IFN-γ). Subchronic CORT pretreatment greatly (up to 500 fold) augmented the neuroinflammatory response to LPS and DFP across brain regions. While PB and DEET failed to affect DFP-induced neuroinflammation, they did reduce inflammatory cytokine expression systemically. Interestingly, the priming of the CNS inflammatory response by CORT was preserved in serum but not liver in LPS-exposed mice. The exacerbated inflammatory response seen in our model is consistent with recently published data in which veterans suffering from GWI have an exaggerated inflammatory response to exercise stress that is specific to this illness (Broderick et al., 2013). Systemic indices of disease are of paramount importance as we investigate GW Illness in veterans still suffering with this disease. (Supported by CDMRP W81XWH-09-2-0098)